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Molecular preservation in Late Cretaceous sauropod dinosaur eggshells

M. H. Schweitzer[1,2,*], L. Chiappe[3], A. C. Garrido[4], J. M. Lowenstein[5] and S. H. Pincus[6,7]

[1]*Departments of Microbiology and Museum of the Rockies, Montana State University, Bozeman, MT 59717, USA*
[2]*Department of Marine, Earth and Atmospheric Sciences, North Carolina State University, Raleigh, NC 27695, USA*
[3]*Natural History Museum of Los Angeles County, Los Angeles, CA 90007, USA*
[4]*Museo Carmen Funes, Avenida Córdoba 55 (8318)-Plaza Huincul, Provencia del Neuquén, Republic of Argentina*
[5]*Department of Nuclear Medicine, University of California, San Francisco, CA 94115, USA*
[6]*Department of Microbiology, Montana State University, Bozeman, MT 59717, USA*
[7]*Research Institute for Children, Children's Hospital and LSU Health Sciences Center, New Orleans, LA 70118, USA*

Exceptionally preserved sauropod eggshells discovered in Upper Cretaceous (Campanian) deposits in Patagonia, Argentina, contain skeletal remains and soft tissues of embryonic Titanosaurid dinosaurs. To preserve these labile embryonic remains, the rate of mineral precipitation must have superseded post-mortem degradative processes, resulting in virtually instantaneous mineralization of soft tissues. If so, mineralization may also have been rapid enough to retain fragments of original biomolecules in these specimens. To investigate preservation of biomolecular compounds in these well-preserved sauropod dinosaur eggshells, we applied multiple analytical techniques. Results demonstrate organic compounds and antigenic structures similar to those found in extant eggshells.

Keywords: palaeoimmunology; dinosaur; immunohistochemistry; eggshell; embryo; histology

1. INTRODUCTION

The recent discovery of titanosaurid dinosaur eggs containing embryonic remains in Argentina shed light on the phylogeny (Chiappe *et al.* 1998, 2000, 2001), development (Chiappe *et al.* 2001) and behaviour (Chiappe *et al.* 2004) of this important group of extinct vertebrates. Dinosaur eggs from this locality preserve not only embryonic bone material, including articulated skulls (Chiappe *et al.* 2001), but also the first reported fragments of embryonic dinosaur skin, preserved not simply as impressions, but in three dimensions (Chiappe *et al.* 1998; figure 1). The preservation of embryonic soft tissues is highly significant, because it indicates unique depositional and geochemical conditions that resulted in rapid and complete mineralization of these very labile soft tissues before degradation could occur. These taphonomic conditions extend to the eggshell, and as a result, both eggs and their contents represent a unique opportunity to elucidate taphonomic conditions resulting in exceptional preservation. We define exceptional preservation as a taphonomic mode preserving soft tissues, original biomolecules or their altered fragments, original mineralogy and/or other features normally lost during diagenesis. The exceptional morphological preservation of these specimens encouraged analytical experiments designed to determine if this preservation extended to the molecular level.

The microscopic, molecular and elemental analyses we describe herein are limited to eggshell, rather than embryonic remains, because of the destructive nature of these analyses. Eggshell was more prevalent (though less morphologically informative) than embryonic remains, and serves as a proxy for the preservational state of the rarer embryonic bone and skin. Immunological data support the presence of at least some immunogenic molecules in these fossil shells that, although altered, share characteristics in common with organic material in extant eggshells.

2. GEOLOGIC SETTING

Sauropod eggs and embryos were recovered from the Anacleto Formation (uppermost unit of Neuquén Group) near Auca Mahuevo (Chiappe *et al.* 1998, 2000, 2001, 2004). This unit has been assigned by Legarreta & Gulisano (1989) to middle Campanian, a date recently confirmed by Dingus *et al.* (2000) for Auca Mahuevo outcrops using palaeomagnetic data.

Sedimentological evidence suggests a palaeoenvironment characterized by mixed-load meandering rivers developed over a low-gradient, extensive floodplain. Thick overbank deposits show signs of recurrent, periodical flooding events that buried the nests and contributed to preservation of the eggs over time. Pedogenic caliche levels and heavy phytoturbation (root-traces) influence the overbank and older channels deposits, indicating well-developed palaeosols and abundant vegetation. However, the only fossil evidence of this vegetation is in the form of small- and medium-sized plants.

Caliche formation and vertisol development, together with the presence of evaporitic sediments in the sequence, suggest warm and relatively arid palaeoclimatic conditions with a pronounced alternation of wet and dry seasons (Esteban & Klappa 1983; Goudie 1983; Bridge 1984; Jerzykiewicz & Sweet 1987).

* Author for correspondence (schweitzer@ncsu.edu).

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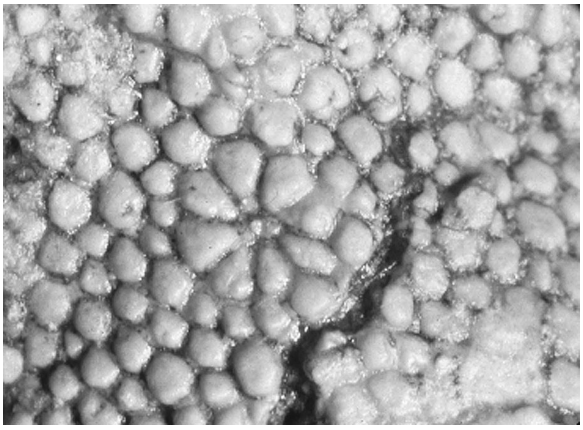

Figure 1. Transmitted light microscopy image of skin fragment from sauropod embryo. Intricate scale pattern is clearly visible.

The eggs are arranged in clutches distributed across at least four stratigraphic layers in an 85 m thick sequence of sandstone, siltstone and mudstone (Chiappe *et al.* 2000, 2004). In each horizon, clutches typically contain 15–30 eggs arranged in layers, and are found in depressions underlain by either mudstone or sandstone. After the eggs were deposited, flooding events caused the depressions to be filled with silty mud that that settled out of suspension. Channel and crevasse-splay deposits, represented by thin sandstone lenses that interfinger laterally with mudstone units, are found within some regions of the egg-bearing intervals. The clutches are located within silty, reddish-brown mudstone units with slickensided surfaces that indicate vertisol development (Chiappe *et al.* 2000). The depressions containing several clutches truncate bedding and sedimentary structures, suggesting excavation by the adult sauropod (Chiappe *et al.* 2004).

3. MATERIAL AND METHODS

See Electronic Appendix.

4. RESULTS

(a) *Microscopy*

It is beyond the scope of this paper to undertake a complete discussion of eggshell microstructure, ootaxonomy and its interpretations. The literature is replete with examples, classification and analyses of extant and fossil vertebrate eggshell structure. However, briefly, the shell microstructure of these eggs is consistent with Megaloolithidae (Mikhailov 1991, 1992; Hirsch 1996; Carpenter 1999; Khoring 1999), an ootaxon traditionally associated with Sauropoda (Hirsch 1996). Lack of definitive embryonic remains associated with shells has made that assignment tentative until the discovery of these specimens with embryonic remains (Chiappe *et al.* 1998).

The quality of morphological preservation of these fossil shells is demonstrated using transmitted light and scanning electron microscopy (figure 2*a*,*b*). External shell morphology consists of closely spaced knobby projections, or tubercles (T; figure 2*a*), with straight, unbranched pores (P) for gas exchange arranged in the depressions between tubercles (Chiappe *et al.* 1998). Radial ground sections of some specimens show intact shell units (figure 2*a*) as vertical columns with roughly parallel, distinct margins, showing minimal alteration of original structure. Fine laminations seen in transmitted light microscopy (figure 2*a*, arrowheads) represent periodic accretion of mineral upon the organic matrix of the shell during biomineralization, tracking outward growth of the shell from nucleation sites within the external shell membrane (SM) (Vianey-Liaud *et al.* 1994; Carrino *et al.* 1996; Chiappe *et al.* 1998; Carpenter 1999; Grellet-Tinner *et al.* 2004). Erosion seen at the base of the shell units (figure 2*a*,*b*) may be the result of demineralization by the embryo or of subsequent early diagenetic dissolution. The borders of these eroded areas are lined with a dark brown, non-translucent material consistent with diagenetically altered organics. In addition, apparent organic cores (OC, figure 2) can be seen embedded in a laminated material (SM) that lies immediately internal to the mineralized shell, features confirmed in electron microscopy (figure 2*b*). Based upon location, structure and comparison with extant taxa (e.g. figure 2*c*), we hypothesize that this material may represent remnants of preserved SM, permineralized by calcite precipitation during fossilization (Jackson *et al.* 2002).

This membrane-like feature is also visible in figure 3, which shows a cross-section of a double-shelled egg. These pathological eggs, similar to other described pathological dinosaur eggs (Zelenitsky & Hills 1997; Hirsch 2001), were quite common in this nesting horizon. The fibrous material interpreted as membrane (M) divides the two sequentially biomineralized eggshell layers (Jackson *et al.* 2002, 2004) consistent with occurrences in extant birds (Jackson & Varrichio 2002 and references therein) and reptiles (e.g. Ewert *et al.* 1984).

5. ELEMENTAL ANALYSES

Energy dispersive X-ray (EDX) elemental analyses shows that element distributions in both sauropod shell and membrane are within the range of variation seen in comparable extant samples (figure 4*a–d*; table 1), suggesting minimal diagenetic alteration. Freshly fractured surfaces of sauropod eggshell (figure 4*a*) also show that the elemental content of the shell differs from that of the membrane (figure 4*b*) in both weight per cent and atomic per cent (table 1). These compositional differences were most probably present during formation of the egg, as they are consistent with those observed in extant specimens (figure 4*c*,*d*). The membrane is enriched in traces of Mn, Mg, Al, Si and K, elements that are also present in one or more examples of extant SMs, but not present in the shell itself. Similar distributions of minor elements are seen in extant shell and membrane from chicken, ostrich and crocodile (table 1), suggesting that these distributions reflect original composition rather than diagenetic artefact. Ratios of the most common elements in both shells and membranes (Ca, C, O) show that extant ostrich shell values are more similar to sauropod shells than to other extant shells in both atomic and weight percentages. These values for membranes across taxa, however, demonstrate wider variation, and reflect the greater degree of mineralization of sauropod membrane relative to other taxa. Weight per cent ratios are depicted graphically in figure 5.

(a) *Extraction of organic components*

Eggshell is a composite material, consisting of calcite mineral (in most hard-shelled eggs) deposited on a pre-existing

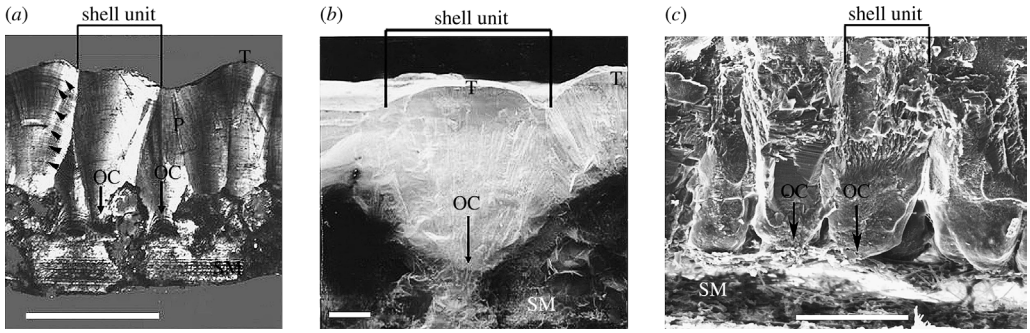

Figure 2. (*a*) Tangential ground section of sauropod eggshell, showing external topography in the form of small knobs or tubercles (T), shell units, accretion lines indicating periodic mineral deposition (black arrowheads), and organic cores (OC, arrows) embedded within shell membrane (SM). A pore (P) extends to the outer surface for oxygen exchange. Scale bar, 1 mm. (*b*) Scanning electron micrograph (SEM) of sauropod shell in tangential section, showing external tubercles (T), shell units, membrane (SM) and organic cores (OC). Scale bar, 200 μm. (*c*) SEM of extant domestic fowl. Shell units are visible, and organic cores (OC, arrows) are seen embedded in the SM. Scale bar, 100 μm.

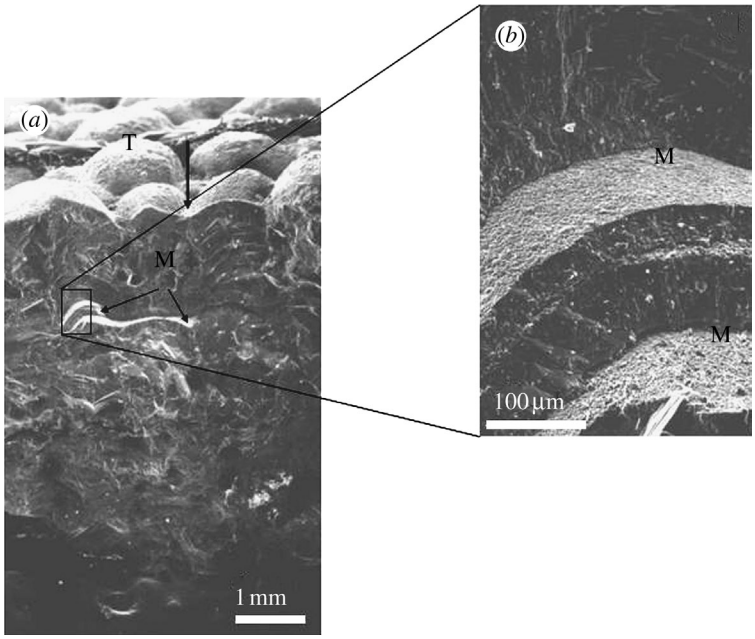

Figure 3. Scanning electron micrographs of double-layered (pathological) sauropod eggshell in tangential section. (*a*) Tubercles (T) delineate the external surface. Preserved shell membrane (M) in between and separating the two layers of shell. Arrow indicates a pore in the depressed region between tubercles connecting exterior to interior of shell. (*b*) Magnification of area shown in (*a*), illustrating the difference in texture between the membrane layer (M) and the surrounding shell matrices. Scales are as indicated.

protein-derived matrix (Dennis *et al.* 1996, 2000; Arias & Fernandez 2001). Analysis of the organic fraction requires chemical extraction. To prepare the sauropod eggs for extraction, they were subjected to surface grinding to remove external contamination. Surface grinding and subsequent crushing of the sauropod shells released a very strong petroliferous odour arising only from the shell, and not from the surrounding sediments or precipitated calcite matrix. This petroliferous odour grew progressively stronger during demineralization of fossil eggshell fragments, but did not accompany demineralization of either extant material or mineral precipitate adjacent to the shell. After grinding to remove surface contamination, fossil and extant eggshells and mineral precipitate were incubated with a guanidinium isothyocyanate buffer to extract organic components, followed by extensive dialysation and subsequent lyophilization (see Electronic Appendix, methods).

The dialysed supernatants of fossil shell extracts did not lyophilize to completion but left a thick, oily residue with a brownish hue. Chicken eggshell likewise left an oily but colourless residue. Conversely, while the sediments had a greater mass to begin with than any eggshell, both sediment extracts and buffer controls had minimal residue after lyophilization, and what remained was colourless, and crystalline rather than oily.

(b) *Immunological analyses*

Sera obtained from rabbits immunized with chemically extracted fossil and extant eggshells were tested for reactivity against various antigens using enzyme linked immunoassay (ELISA). Figure 6 graphically depicts the results of a representative ELISA. Pre-immune sera (drawn from the host before immunization, white, dark grey bars) show no significant reactivity with any antigen tested by ELISA assays. Sera from rabbits immunized with either chicken (light grey bars) or sauropod (black bars) shell extract demonstrated antibody binding to extracted chicken shell and to ovalbumin, an abundant protein in eggshell organic

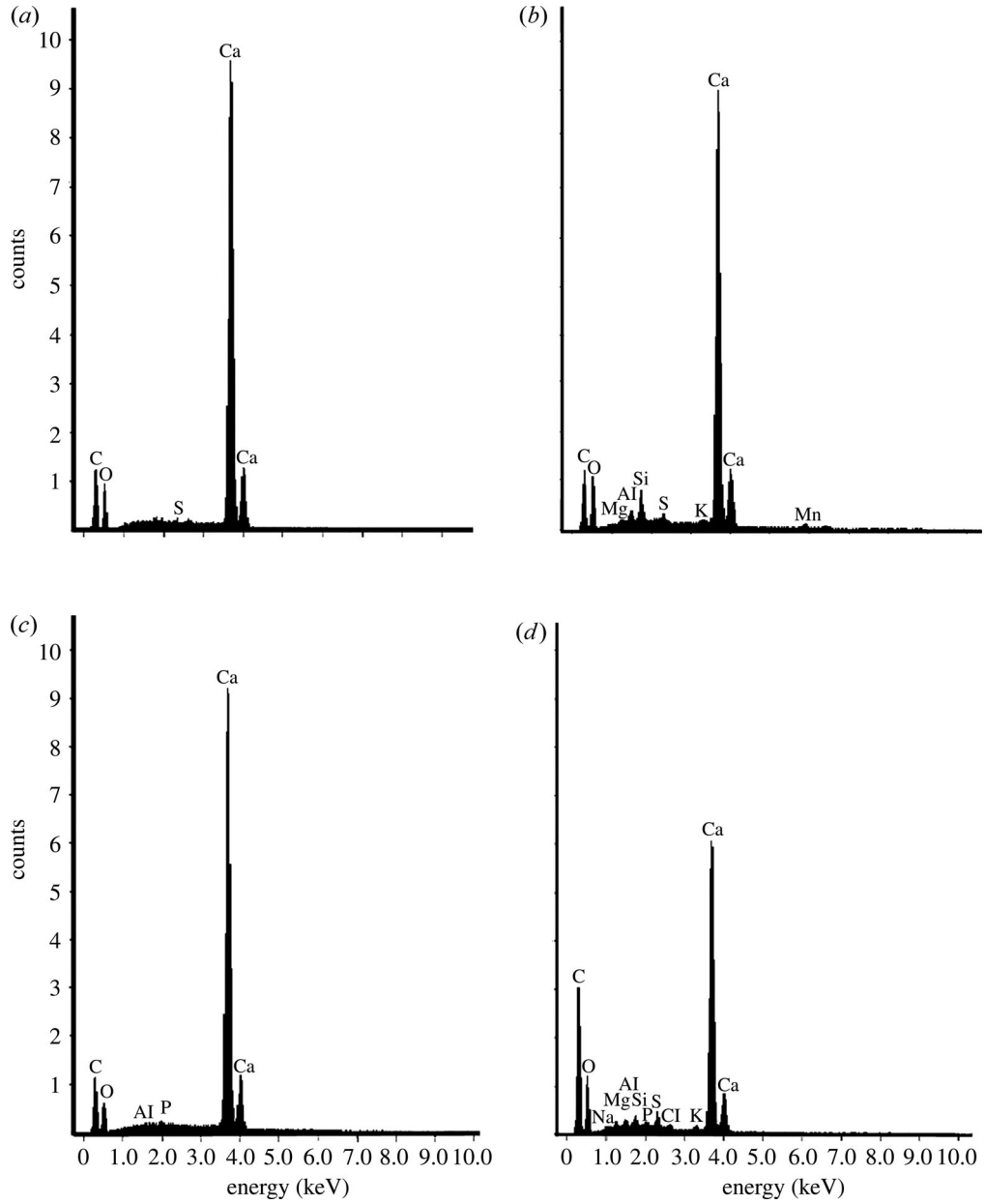

Figure 4. Energy dispersive X-ray (EDX) elemental profile taken through clean fracture of sauropod shell (*a*) and membrane (*b*), compared with extant ostrich shell (*c*) and membrane (*d*). Element profiles are similar, with a similar content and distribution of trace elements in the membranes not present in the shells. Carbon is higher in the ostrich membrane than the shell, indicating a greater organic content. More labile elements are missing from the fossil analyses (e.g. Cl and Na) as would be expected.

matrices and SMs (Nys *et al.* 1999, 2001; Panheleux *et al.* 1999; Arias & Fernandez 2001) significantly above background levels. However, only dinosaur shell antibodies reacted above background to dinosaur shell antigen by this method. While reactivity was greatly decreased from that seen with chicken shell antigen, it was elevated above both pre-immune serum and all sera tested against extracted calcite.

To demonstrate localization of antigens present in fossilized specimens, and to demonstrate that the antibodies are specific for endogenous antigens and not materials that infiltrated during or after the mineralization process, antisera and pre-immune sera were incubated with ground sections of sauropod, chicken and crocodile eggshell. As in ELISA, pre-immune sera did not react with any of the eggshells tested, but immune sera raised against both chicken and dinosaur shell extracts demonstrated specific and localized reactivity (figure 7). Both sera have similar binding patterns, whether on sauropod (figure 7*a*,*b*) or chicken eggshell (figure 7*c*,*d*) ground sections. Relatively homogeneous distribution of fluorescent signal across the shell (palisade layer; Jackson & Varrichio 2002) reflect localization of matrix proteins in extant material (figure 7*c*,*d*). Binding of both dinosaur antibodies and chicken antibodies is greater in regions high in protein content (e.g. external shell, corresponding to thin layer of organic cuticle, and interior of shell corresponding to mammillary layer (ml) and OC. Similar distribution is seen in ground sections of chicken shell. Very bright and patchy distribution may reflect non-specific adherence of antiserum to roughened regions of the dinosaur shell or infilling mineral, but in most regions corresponding to calcite precipitate (CP, arrows), antibody binding is decreased over the rest of the shell, and signal is localized to the shell matrix, membrane, and OC.

Table 1. Table showing weight and atomic per cent values for element distribution.

element	sauropod shell		chicken shell		crocodile shell		ostrich shell	
	wt%	at%	wt%	at%	wt%	at%	wt%	at%
C	16.58	26.16	17.32	26.11	20.07	30.98	15.97	25.75
Ca	46.24	21.87	35.84	16.19	37.76	17.47	42.53	20.55
O	43.5	51.54	50.81	57.5	44.44	51.51	44.23	53.54
Mn								
Mg			0.26	0.2	0.05	0.04		
Al							0.02	0.02
Si								
P							0.24	0.15
S	0.32	0.19						
Cl	0.44	0.23						
K								
Ca/C	2.79	0.84	2.07	3.62	1.88	0.56	2.66	0.80
O/C	2.62	1.97	2.93	2.20	2.21	1.66	2.77	2.08
O/Ca	0.94	2.35	1.42	3.55	1.18	2.95	1.04	2.61
	sauropod membrane		chicken membrane		crocodile membrane		ostrich membrane	
	wt%	at%	wt%	at%	wt%	at%	wt%	at%
C	16.21	25.03	25.36	36.84	44.93	53.22	35.84	46.03
Ca	42.94	19.87	26.02	11.32	23.12	8.21	31	11.93
O	44.98	52.14	46.43	50.62	40.6	36.1	41.41	39.92
Na							0.34	0.23
Mn	0.92	0.31						
Mg	0.24	0.18	0.14	0.1			0.27	0.17
Al	0.7	0.48	0.09	0.06	1.5	0.79	0.47	0.27
Si	1.86	1.23			0.66	0.33	0.64	0.35
P							0.5	0.25
S	0.81	0.47	1.8	0.98	2.47	1.1	1	0.48
Cl			0.15	0.07	0.62	0.25	0.4	0.18
K	0.6	0.28					0.48	0.19
Ca/C	2.65	0.79	1.03	0.31	0.51	0.15	0.86	0.24
O/C	2.77	2.08	1.83	1.37	0.90	0.68	1.16	0.85
O/Ca	1.05	2.62	1.78	4.47	1.76	4.40	1.34	3.57

Finally, both antisera were tested against ground sections of crocodile egg, to test antigen similarity and distribution from that seen in extant birds. Figure 8 shows that antibodies to chicken shell react strongly with both crocodile shell and the SM (figure 8*a*), and fluorescent signal concentrates in the ml, which is rich in organics. Although reactivity of dinosaur antibodies to crocodile shell is significantly less, binding can be seen in the lower portions of the ml and innermost portion of the SM, regions similar to the pattern seen with chicken shell serum. To further demonstrate specificity, dinosaur antibodies were incubated with an excess of dinosaur shell extract to block the binding sites on the antibodies that are specific to sauropod antigen. The antibodies specific to sauropod antigens would then be unavailable to bind antigen in the crocodile shell that was similar to sauropod, but would leaving other, non-specific antibodies free to bind. Additionally, if antibodies raised against sauropod antigens were not specific for those antigens, the more concentrated antigens present in the extant shell would out-compete the dinosaur inhibitor, and binding would be unaffected.

The inhibited antibodies were then incubated with the crocodile shell as described. The binding pattern seen in figure 8*b* disappears when specific antibodies are blocked

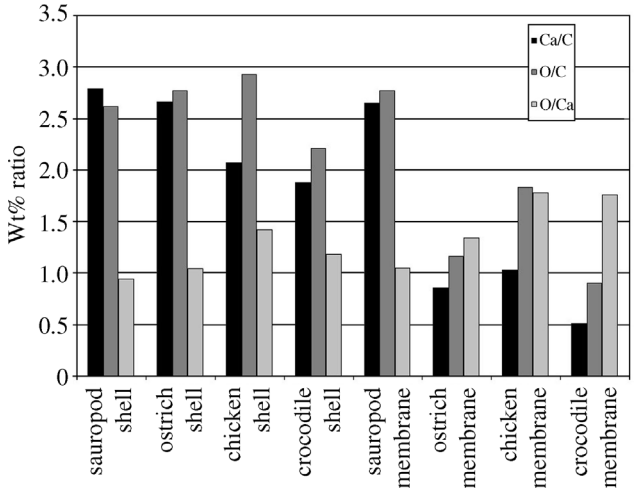

Figure 5. Graphic display of elemental (EDX) data, showing relative values of Ca/C, O/C and O/Ca. These ratios are virtually identical for ostrich and sauropod shell, while ratios obtained from other extant shells vary significantly in these values. Ca/C and O/C values are much higher in sauropod membrane than any extant membrane studied, consistent with a greater degree of mineralization. Extant SMs show greater O/C than Ca/C, a trend also seen in sauropod membrane.

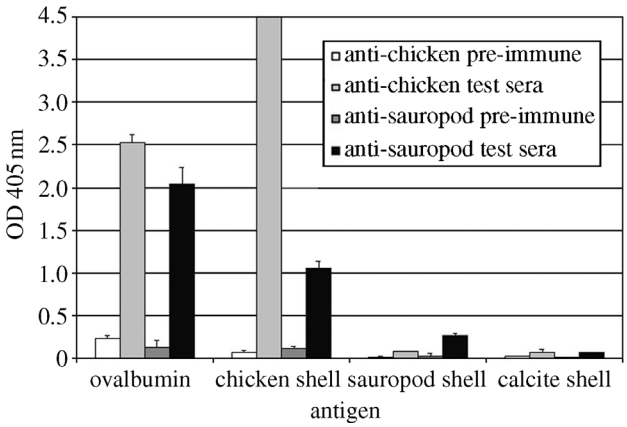

Figure 6. Representative ELISA showing reactivity of pre-immune (white, dark grey bars) and post-immunization (light grey, black) test sera against multiple antigens. Both test sera show significant reactivity with purified ovalbumin, a predominant constituent of extant eggshell matrix and membrane (Nys *et al.* 2001). Chicken eggshell antibodies react significantly above controls to extracted sauropod and chicken eggshell antigens. Only sauropod antiserum reacts with saruopod shell extracts. No reactivity is demonstrated by any sera with extracted calcite mineral.

(figure 8*c*). This supports specificity as well as molecular similarity between antigenic components in the shells of both taxa, and demonstrates the shared nature of some of the components of crocodile shell matrix with those of sauropod eggshells.

6. DISCUSSION

The cross-reactivity of chicken antiserum with sauropod shell components, and sauropod antiserum with chicken eggshell was demonstrated by two different assays (figures 6 and 7). These results indicate molecular similarity of shell-derived immunogens. This point is further emphasized by the localization of antibody binding (figures 7 and 8), suggesting that similar biochemical structures have comparative sites of localization in both chicken and sauropod eggshells. Both cross-reactivity and pattern of localization provide evidence that the antisera are detecting endogenous sauropod antigens.

To propose that this pattern arises from contamination would require that the contaminant, molecularly similar to eggshell proteins, infiltrated the shell and distributed in a pattern similar to the distribution of extant shell organics. Additionally, the reactivity of both antisera to ovalbumin (figure 6) would require that the contaminant also be present in this biochemically purified protein. A far more parsimonious explanation is that ovalbumin is one of the cross-reactive materials recognized by the antisera, and that sauropod eggshells retain fragments of original, immunogenic and antigenic organic material which are homologous with some components in extant shell matrices.

We have shown that in these well-preserved fossils, the molecular structure of endogenous antigens may be preserved for more than 70 Myr. The results show that antigens preserved within fossil material retain immunogenicity, and that antisera made against both extant and fossil materials can detect these antigens in fossils. Although the exact chemical nature of the antigenic material has not been demonstrated, it has retained similarities to extant native material in regard to those structures recognized by the immune system.

Evidence supporting the preservation of endogenous biomolecules in the pre-Cenozoic fossil record has generally been met with scepticism, because it is assumed that primary organic molecules cannot withstand the alterations and breakdown that occur during diagenesis (e.g. Bada 1985; Runnegar 1986; Logan *et al.* 1991; Lindahl 1993). Laboratory experiments designed to approximate molecular diagenesis apply physical and chemical parameters not normally encountered in nature (e.g. pH = 1, $T \geq 300$ °C) and do not account for the protective effects of mineral association (e.g. Weiner *et al.* 1989; Glimcher *et al.* 1990; Sykes *et al.* 1995). Therefore, their utility as a proxy for diagenetic processes at the molecular level in naturally preserved samples is somewhat limited. We propose that, while such experiments proved useful information regarding possible degradation pathways, and some recent findings indicate that protein persistence has been underestimated (Collins *et al.* 2000), a more *direct* test of molecular longevity is the application of multiple and varied analyses for endogenous molecular components to extraordinarily preserved fossils. We propose that exhaustive investigation of a variety of these exceptional assemblages, from a spectrum of depositional and diagenetic environments, is an important way of assessing biomolecular preservation, because normal diagenetic processes are both slower and less extensive than those simulated in laboratory experiments.

Despite scepticism regarding the premise of long-term molecular survival based on laboratory experiments and predictions of molecular kinetics, many studies have shown that amino acids, short peptides, and amino sugars can persist within fossils over a wide geological age distribution (e.g. Weiner *et al.* 1976; Westbroek *et al.* 1979; Armstrong *et al.* 1983 and references therein; Lowenstein 1981, 1985; Ostrom *et al.* 1990; Collins *et al.* 1991; Gurley *et al.* 1991; Muyzer *et al.* 1992; Stankiewicz *et al.* 1997*a*,*b*, 1998; Schweitzer *et al.* 1997*a*,*b*, 1999*a*,*b*, 2002; Poinar *et al.* 1998; Collins *et al.* 1999). Immunological techniques have identified antigenic compounds in fossils of varying ages and from various source taxa (e.g. Rowley *et al.* 1986; Muyzer & Westbroek 1989; Baird & Rowley 1990; Collins *et al.* 1991; Lowenstein & Scheuenstuhl 1991; Child & Pollard 1992; Nerlich *et al.* 1993; Franc *et al.* 1995; Borja *et al.* 1997; Schweitzer *et al.* 2002) including Cretaceous fossils (Collins *et al.* 1991; Muyzer *et al.* 1992; Schweitzer *et al.* 1997*b*, 1999*a*,*b*).

To preserve fossils in an exceptional manner requires early cessation of diagenetic processes, and is attributable to unusual physical and chemical conditions from death through diagenesis, and minimal alteration of fossil material at the macro and microscopic levels. This, in turn, is correlated with the preservation of endogenous biomolecules, fragments of molecules, or biomarkers (i.e. altered molecular fragments that can be traced to the original source, e.g. Hagelberg & Clegg 1991; Hedges 2002; Schweitzer *et al.* 2002). Because molecular preservation has been correlated with morphological and microstructural preservation (e.g. Hagelberg & Clegg 1991; Marota & Rollo 2002), optimizing the search for endogenous organic components within fossil specimens should involve careful selection of fossil specimens for

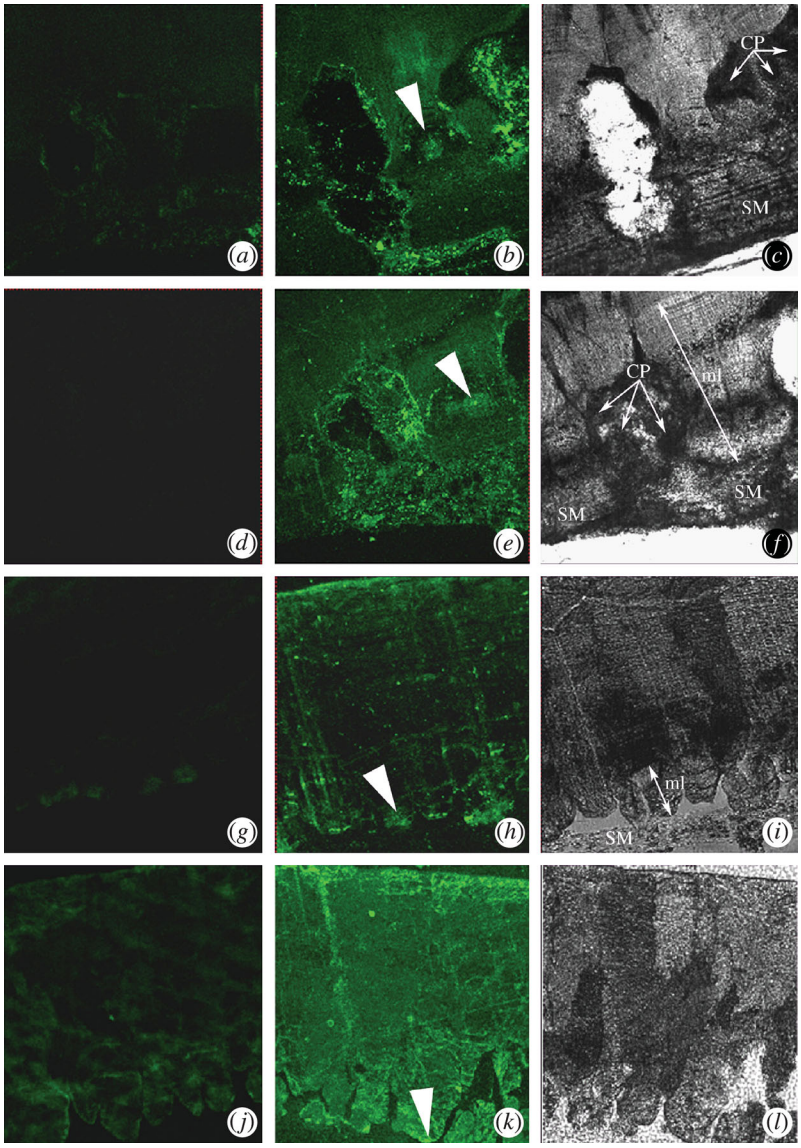

Figure 7. Immunohistochemical localization of antigens in ground sections of extant and fossil eggshell. First two columns are imaged using confocal fluorescence microscopy, third column is transmitted light image. (*a*),(*d*) Sauropod shell, and (*g*),(*j*) chicken shell, incubated with pre-immune sera. (*b*) Sauropod shell exposed to antisauropod antiserum. (*e*) Sauropod shell exposed to chicken antiserum. (*h*) Chicken shell exposed to antisauropod antiserum. (*k*) Chicken shell exposed to antichicken antiserum. (*c*),(*f*),(*i*),(*l*) same sample as (*b*),(*e*),(*h*),(*k*) correspondingly, visualized in transmitted light. Fluorescent label (green) corresponds to location of antibody–antigen complexes. Dark regions show no specific antibody binding. All data were collected under identical exposure and integration conditions. Intensity correlates with degree of antibody binding, pattern of fluorescent distribution corresponds with location of components recognized by antibody. White arrowheads show location of organic cores (OC) within mammillae. CP, arrows show regions of calcite precipitation between mammillae of sauropod shell; ml, mammillary layer of shell; and SM, shell membrane.

study. Analyses of such extraordinary samples are a direct test of survivability of biomolecules.

However, molecular analyses of fossils present unique challenges. In part, this is because chemical modifications occur during diagenesis (Mycke & Michaelis 1985; Rafalska *et al.* 1991; Poinar *et al.* 1998). These modifications include breaking peptide bonds, removal or alteration of original amino acid side chains, and cross-linking of peptide fragments to other organic degradation products, a process that makes organic material insoluble and difficult to separate into constituent compounds (Macko & Engel 1991; Poinar *et al.* 1998). Condensation reactions along this pathway may result in the formation of hydrocarbons from proteinaceous precursors. Because these products are hydrophobic and because the original organics are contained within a biomineralized matrix (Weiner *et al.* 1989; Glimcher *et al.* 1990; Sykes *et al.* 1995), the chances of retention of organic material that contains remnants, however altered, of the original compounds are greatly increased. The strong petroliferous odour released upon decalcification of the eggshell supports this pathway of degradation in these eggshells. A lipid-containing molecular complex of degraded organics and antigenic material may explain some of the anomalies seen in our data. For example, low reactivity of antibodies with dinosaur antigen by ELISA may be owing to insufficient immobilization of antigen resulting from lipid or hydrocarbon mixing with the antigens.

Finally, while the organic material extracted from dinosaur eggshells shows characteristics consistent with extant material similarly derived, it is recognized that the antigenic material may or may not be derived from

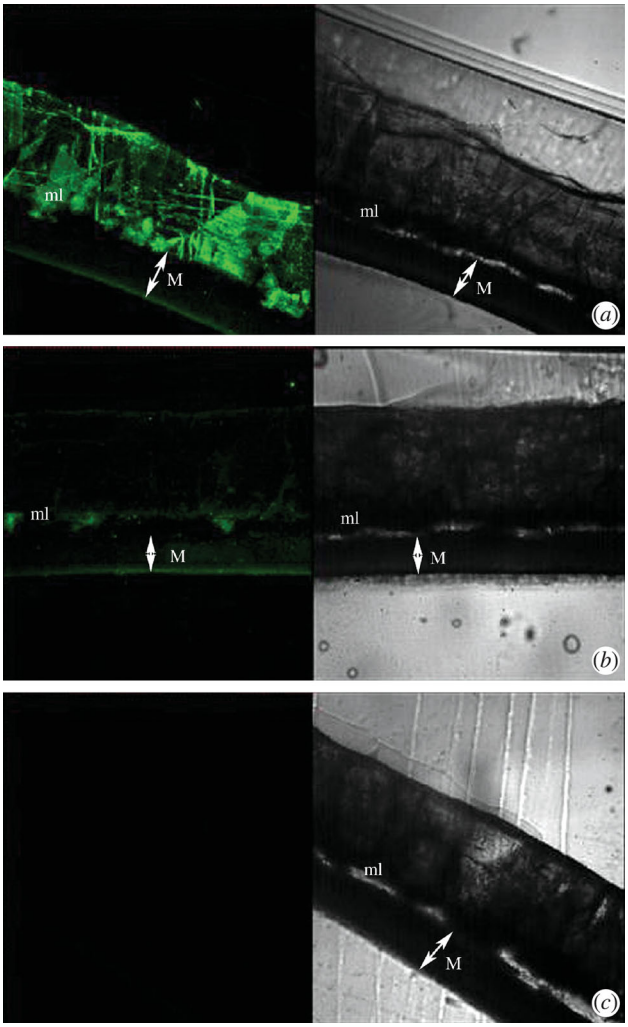

Figure 8. Immunohistochemical localization of antigens in ground sections of crocodile eggshell probed with antichicken and antidinosaur shell antibodies. Left panels are imaged using confocal fluorescence microscopy, right panels are transmitted light images. (*a*) chicken shell antiserum shows strong reactivity throughout the shell matrix, concentrated in the mammillary layer (ml) and the innermost border of the shell membrane (SM). (*b*) Sauropod shell antiserum shows reduced reactivity of the antiserum to components of crocodile shell matrix, and reactivity is mostly concentrated in the mammillary layer (ml) and the outer SM. (*c*) Antiserum incubated with excess sauropod extract prior to exposure to crocodile shell to inhibit binding of sauropod-specific antibodies to crocodile epitopes similar in structure to sauropod antigen. See text for discussion.

proteinaceous precursors, and is surely diagenetically altered from its original state. We do not claim here that this material represents complete proteins. Indeed, epitopes are known to be only a few amino acids in length (Child & Pollard 1992); therefore, it is possible that antigenic response may be owing to selective preservation of a few peptides, or even altered, fossilized derivatives of peptides.

The antisera that we have prepared may be used to purify the antigenic material through affinity isolation techniques. It may then be possible to perform further biochemical analyses upon these materials, thus offering the promise of subjecting dinosaur tissues to modern molecular techniques, including amino acid sequencing (Schweitzer *et al.* 2002).

These eggs, containing such fragile and labile elements as embryonic bone and fragments of embryonic skin, attest to unusual taphonomic and diagenetic conditions and provide an opportunity to expand the correlation between unusual morphological preservation and the presence of endogenous molecules.

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